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Infection of a Human Retinal Pigment Epithelial Cell Line With Human Herpesvirus 6 Variant A

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A retinal pigment epithelial (RPE) cell line (K-1034) was examined for its susceptibility to human herpesvirus 6 variant A (HHV-6A). Exposure of K-1034 cells to HHV-6A induced the formation of multinucleated giant cells, which was suppressed by an inhibitor of viral DNA synthesis. In the giant cells, herpesvirus nucleocapsids were demonstrated by electron microscopy and the viral glycoprotein B was detected by immunofluorescence assay. These results indicate that K-1034 cells are susceptible to HHV-6A and suggest that HHV-6A has an ability to directly destroy epithelial cells. ***J. Med. Virol. 53:105–110, 1997.***

KEY WORDS: retinal pigment epithelial cells; human herpesvirus 6; lymphotropic betaherpesvirus

INTRODUCTION

Human herpesvirus 6 (HHV-6) is a recently discovered lymphotropic betaherpesvirus [Josephs et al., 1986; Salahuddin et al., 1986; Ablashi et al., 1988a] and is widely distributed in the general population [Harnett et al., 1990]. HHV-6 can be classified into two groups, variant A (HHV-6A) and variant B (HHV-6B) [Ablashi et al., 1993]. HHV-6B is a causative agent of exanthem subitum [Yamanishi et al., 1988], however, association of HHV-6A with clinical entities is still unclear.

Although HHV-6A prefers CD4-positive T cells as its host to replicate, the virus can infect other cells including B lymphocytes, monocyte/macrophage, natural killer cells, megakaryocyte and glial cells [Ablashi et al., 1987; Downing et al., 1987; Lusso et al., 1987; Ablashi et al., 1988a; Lusso et al., 1988, 1993]. About HHV-6A infection of human epithelial cells, only one report has been described. In the report, HPV-immortalized or transformed cervical epithelial cells were permissive for HHV-6A [Chen et al., 1994]. HHV-6A DNAs and early-late proteins were detected in the infected cervical cells. However, no cytopathic effect (CPE) was observed in the cervical cells and viral antigen detection in the cervical cells were restricted in the acute phase of infection. These results are insufficient for determining whether HHV-6A has an ability to directly cause damage to epithelial cells. In this report, we studied susceptibility of a human retinal pigment epithelial cell line (K-1034) as part of a series of studies to clarify pathogenicity of HHV-6A to epithelial cells.

METHODS AND MATERIALS

HHV-6A

K-1034 cells were obtained from Dr. K. Kigasawa. The cells retain epithelial morphology [Kigasawa et al., 1994], acid phosphatase activity [Kigasawa et al., 1995], and abilities to synthesize melanin and express kelatin 18 antigen (unpublished data), although they have been passaged more than 100 times. The U1102 strain of HHV-6A [Salahuddin et al., 1986] and its host T cell line, HSB-2 [Ablashi et al., 1988b], were kindly provided by Drs. K. Kondo and K. Yamanishi. These cell lines were cultured at 37°C in Dulbecco's modified minimum essential medium supplemented with 10% of fetal bovine serum and 100 mg/ml of kanamaycin (the growth medium).

First, we exposed K-1034 cells to HHV-6A by cocultivating the epithelial cell monolayers with the HHV-6A-infected HSB-2 cells at a 1:1 ratio. The viability of

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the infected HSB-2 cells was assayed by staining the cells with 0.05% nigrosine (Sigma Chemical Co, ST Louis, MO), resulting in that approximately 70% of the cells were viable. The viral antigen-positive rate of the infected HSB-2 cells was approximately 80%, which was examined by indirect immunofluorescence assay (IFA) as described previously [Aoyama et al., 1982]. After fixing in acetone, the HHV-6-infected and uninfected cells were overlayed first with OHV1 [Okuno et al., 1992], a mouse monoclonal antibody against glycoprotein B of HHV-6A and HHV-6B, and second with fluorescein isothiocyanate-conjugated goat antibody against mouse immunoglobulins (Tago Inc., Burlingame, CA). After 24 hr cocultivation, the K-1034 cell monolayers were washed with phosphate buffered saline (PBS) six times to remove the T cells and were incubated at 37°C in the growth medium. Most K-1034 cells, which were cocultivated with the HHV-6A-infected HSB-2 cells, fused on day 3 of infection (Fig. 1B) although no morphologic change was observed in K-1034 cells cocultivated with the uninfected HSB-2 cells (Fig. 1A). The result suggest that K-1034 cells are susceptible to HHV-6A.

Second, we exposed K-1034 cells to HHV-6A by inoculating virus free from the viable cells (VFVC) and examined the giant cell formation of, infectivity titer in, virus particle production in and viral protein synthesis in the epithelial cells. The VFVC stocks of HHV-6A were prepared by storing the infected HSB-2 cells at −80°C in the growth medium containing 10% sucrose. No viable cells were observed after one cycle of freeze and thaw of the stocks. The HHV-6A infectivity titer of the stocks was determined with stimulated cord blood lymphocytes (CBL) as described previously [Simmons et al., 1992] except for the following two points. Interleukin-2 (IL-2) was used to stimulate CBL in addition to phytohemagglutinin (PHA) and HHV-6A infection of CBL was monitored by IFA in place of anticomplement immunofluorescence assay. K-1034 cells were inoculated with HHV-6A at a multiplicity of infection (MOI) of 0.005 50% tissue culture infectious dose ($TCID_{50}$) per cell by seeding the cells with VFVC to quickly form monolayers. After an overnight incubation at 37°C, these cultures were washed with PBS three times and cultivated in the growth medium at 37°C with renewal of the culture medium twice a week.

Cytopathic Effect and Infectivity Titer

The VFVC-exposed K-1034 cells formed giant cells with multiple nuclei (Fig. 1D, 1E, 1F), which were not observed in the mock-infected K-1034 cells (Fig. 1C). The number of nuclei per giant cell was usually from a few to twenty in almost giant cells (Fig. 1D, 1F) and occasionally the nucleus number reached more than eighty (Fig. 1E). The numbers of multinucleated giant cells in the cultures were counted by phase contrast microscopy. Closed circles in Figure 2 show the numbers of the giant cells in the cultures. The giant cells appeared from day 3 of infection and accumulated massively by 7 days after the start of infection. The number of giant cells reached 104 per cm^2 of culture area at the peak, meaning that one giant cell appeared per 770 cells. Degeneration of giant cells occurred thereafter. The number of giant cells gradually decreased with time to less than 1/100 of the maximum number but a very few number of giant cells could be seen after more than six months incubation. HHV-6A infectivity titers were assayed at 3, 7, 14, 21 days after infection (open circles in Fig. 2). The infected cells were harvested by trypsinization and stored at −80°C in a growth medium containing 10% demethyl sulfoxide until infectivity titration. After thawing, ten-fold dilutions of the infected cells were mixed with 1.25×10^5 cells of the PHA and IL-2-stimulated CBL, were incubated for seven days at 37°C, and were examined by IFA as described above. The infectivity was demonstrated only at day 3 of infection with five $TCID_{50}$ per 10^5 cells and did not at days 7, 14, and 21 of infection.

Effect of Inhibitors of Viral DNA Synthesis on HHV-6A-Induced Giant Cell Formation

In this infection system using VFVC, we tested the effect of inhibitors of viral DNA synthesis on HHV-6A-induced giant cell formation (Table I). Ganciclovir (GCV) and phosphonoacetic acid (PAA), which have been reported as effective inhibitors of HHV-6 DNA replication [Agut et al., 1989; Shiraki et al., 1989; Burns et al., 1990; DiLuca et al., 1990], were purchased from Nippon Roche K.K. (Tokyo, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively. The VFVC-inoculated K-1034 cells were incubated for seven days in the presence of GCV or PAA at a concentration of 100 or 50 μg/ml, respectively. The number of multinucleated giant cells in these cultures were counted by phase contrast microscopy and was compared to that in the absence of the drugs. In the absence of inhibitors of viral DNA synthesis, HHV-6A inoculation induced extensive cell fusion. Massive giant cells accumulated by day 7 after inoculation. When GCV or PAA was present in the culture media, the number of the giant cells greatly decreased. Thus, HHV-6A-induced giant cell formation depends on de novo viral DNA synthesis in the infected K-1034 cells.

Electron Microscopic Study

The VFVC-exposed K-1034 cells were analyzed by transmission electron microscopy (Fig. 3). The infected K-1034 cell pellets were prepared by trypsinization and a low speed centrifugation (800 × g, 10 min, 4°C) at day 7 postinfection with VFVC and then examined by transmission electron microscopy (JEOL TEM 1200EX) as described previously [Hoshikawa et al., 1991]. We exclusively analyzed the giant cells in order to increase probability to detect virus particles. As a result, herpesvirus particles could be found in approximately 10% of the giant cells. Intranuclear and intracytoplasmic virus particles were nucleocapsids with and without tegments, respectively. The tegment that

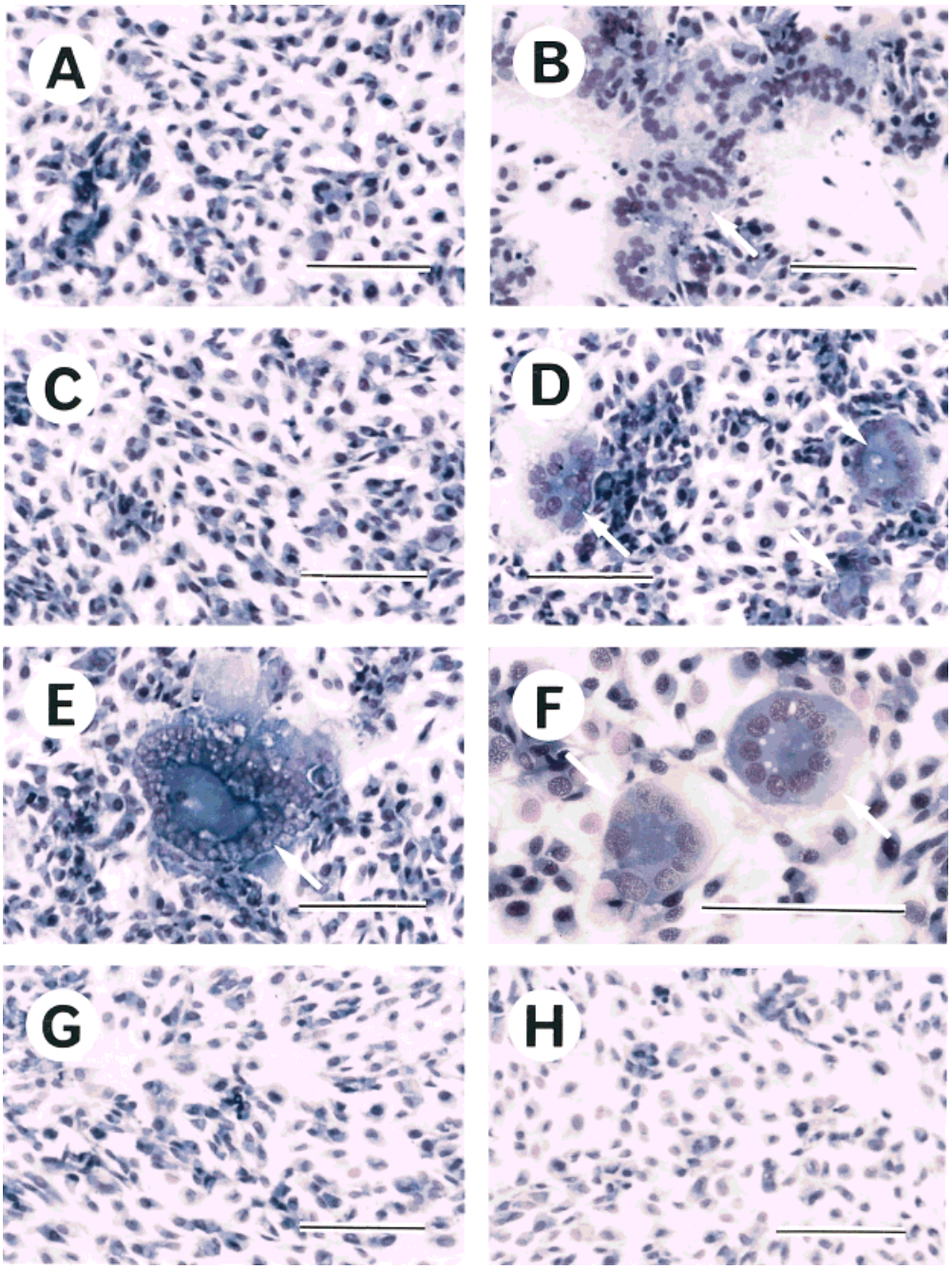

Fig. 1. Giant cell formation due to HHV-6A infection. K-1034 cell monolayers, which were cocultivated with the uninfected HSB-2 cells **(A)** or the HHV-6A-infected HSB-2 cells **(B)** as described in the text, were fixed in methanol at day 3 of infection. The mock-infected **(C)** or VFVC-infected **(D, E, F)** K-1034 cells, which were prepared as described in the text, were fixed in methanol at day 7 of infection. K-1034 cell monolayers, which were cocultivated with the uninfected MT-4 cells **(G)** or the HHV-6B-infected MT-4 cells **(H)** as described in the text, were fixed in methanol at day 14 of infection. The fixed cells were photographed after the Giemsa stain. Arrows indicate multinucleated giant cells. Bars indicate 100 μm.

could be very clearly seen in the intracytoplasmic nucleocapsids was thick and had moderate electron density. This kind of tegment is characteristic of an HHV-6 particle [Yoshida et al., 1989]. The result demonstrates virus particle production in K-1034 cells.

Immunofluorescence Study

Furthermore, viral antigens in K-1034 cells were examined with the VFVC-inoculated epithelial cell cultures (Fig. 4). The VFVC-inoculated cells were fixed in

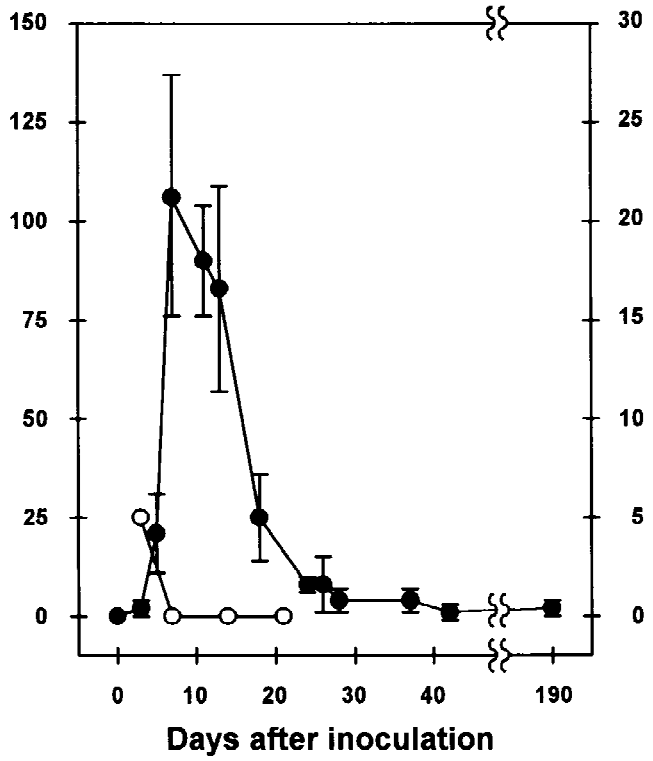

Fig. 2. The number of multinucleated giant cells (closed circles) and the infectivity titer (open circles) in the VFVC-inoculated K-1034 cells K-1034 cell cultures were inoculated with the VFVC stock of HHV-6A at a MOI of 0.005 $TCID_{50}$ per cell and then were examined for cytopathic changes by phase-contrast microscopy. The number of giant cells in a culture area of 0.24 cm^2 was counted at appropriate intervals and the mean number of giant cells per cm^2 was calculated. Bars indicate standard deviations of at least five random countings. HHV-6A infectivity titers in the VFVC-inoculated K-1034 cells were assayed as described in the text.

acetone at day 7 of infection and were examined by IFA as described above. Specific fluorescence was observed in the cytoplasm of multinucleated giant cell and single cell (Fig 4B, 4C, 4D), indicating that HHV-6A antigens can be expressed in the K-1034 cells. Taken together with the result of experiments using viral DNA inhibitors and electron microscopic study, it is concluded that K-1034 cells are susceptible to HHV-6A and the extensive CPE is not caused by mere contact of the epithelial cells to the infected HSB-2 cells but is due to HHV-6A infection of the epithelial cells.

DISCUSSION

A recent study showed that HPV-immortalized or transformed cervical epithelial cells are susceptible to HHV-6A [Chen et al., 1994]. Also in this report, epithelium-derived cells were found to be susceptible to HHV-6A. Thus, epithelial cells seem to be permissive for HHV-6A infection although they have varying degrees of susceptibility.

No generalized morphologic change was observed in the experimental infection of the HPV-immortalized or transformed cervical epithelial cells with HHV-6A, In contrast, HHV-6A infection of K-1034 RPE cells induced extensive cell fusion, leading to formation of multinucleated giant cells and cell degeneration. The virus-induced cell fusion can be of consequence in pathogenicity because the fusion affords a means of spreading infection and directly magnifies damage to the virus-infected tissues. Therefore, the results of the present study may indicate a potential ability of HHV-6A to develop lesions in epithelial cells.

In addition to HHV-6A, we preliminarily tested susceptibility of K-1034 cells to HHV-6B. K-1034 cell monolayers were cocultivated with the HHV-6B-infected MT-4 cells at a 1:1 ratio. The HHV-6B-infected cells were prepared with the HST strain of HHV-6B [Yamanishi et al., 1988] and MT-4 cells [Ablashi et al., 1988b] (gifts of Drs. K. Yamanishi and K. Kondo) and were examined for the cell viability and viral antigen-positive rate as same as the preparation of the HHV-6A-infected HSB-2 cells. The viability and viral antigen-positive rate of the HHV-6B-infected MT-4 cells were similar to those of the HHV-6A-infected HSB-2 cells. In the contrast with the cocultivation experiments with the HHV-6A-infected HSB-2 cells, no generalized morphologic changes were observed in K-1034 cells cocultivated with the HHV-6B-infected MT-4 cells even after 2 weeks incubation (Fig. 1H). Thus, K-1034 cells seems less susceptible to HHV-6B than HHV-6A at least in morphologic changes. HHV-6 is suspected of causing acquired immunodeficiency syndrome (AIDS) associated retinitis [Qavi et al., 1989; Qavi et al., 1992; Reux et al., 1992; Qavi et al., 1994; Qavi et al., 1995; Fillet et al., 1996] and the virus detected in retinal epithelia of AIDS-associated retinitis patients is HHV-6B. HHV-6 positive cells in the retinal lesions are not in retinal pigment epithelia but are mainly located in the ganglion cell layer and in the inner nuclea cell layer. Therefore, it is not likely that higher susceptibility of K-1034 cells to HHV-6A is associated with AIDS-associated retinitis although whether retinal pigment epithelial cells in vivo are more permissive for HHV-6B than K-1034 cells is unknown.

In the VFVC-infected K-1034 cells, the HHV-6A-induced giant cells had accumulated massively by day 7 after the start of infection but thereafter the number of the giant cells decreased with time. The infectivity

TABLE I. Inhibition of HHV-6-Dependent Giant Cell Formation by GCV and PAA*

Drugs	Number of giant cells per cm^2 of culture area ± S.D.
—	77.5 ± 10.0
GCV	0.4 ± 1.3
PAA	0.2 ± 0.9

*K-1034 cells were infected with VFVC at a MOI of 0.005 $TCID_{50}$ per cell and were incubated at 37°C in the absence or presence of GCV (100 μg/ml) or PAA (50 μg/ml). Seven days after inoculation, the number of giant cells in each culture was counted five times at random by phase contrast microscopy and the mean number of giant cells was calculated. S.D. represents the standard deviation of the mean number.

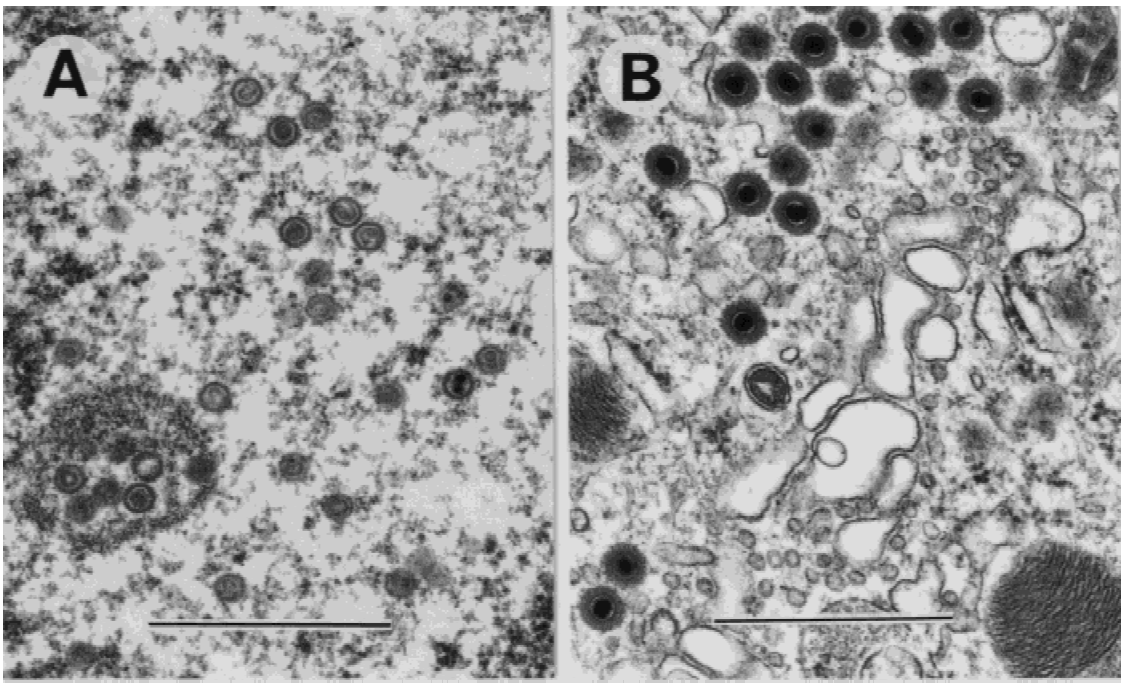

Fig. 3. Transmission electron micrograph of the nucleus **(A)** and cytoplasm **(B)** of a multinucleated giant cell in the VFVC-inoculated K-1034 cell culture 7 days after inoculation. A moderate number of nucleocapsids are observed both in the nucleus and cytoplasm. Bar indicates 1 μm.

titer in the VFVC-infected K-1034 cells was constantly low throughout the period assayed. Electron microscopic study demonstrated virus particles only in approximately 10% of the giant cells. These results suggest that HHV-6A replication in K-1034 cells was considerably restricted. This is consistent with the results of the study using the HPV-immortalized or transformed cervical epithelial cells [Chen et al., 1994].

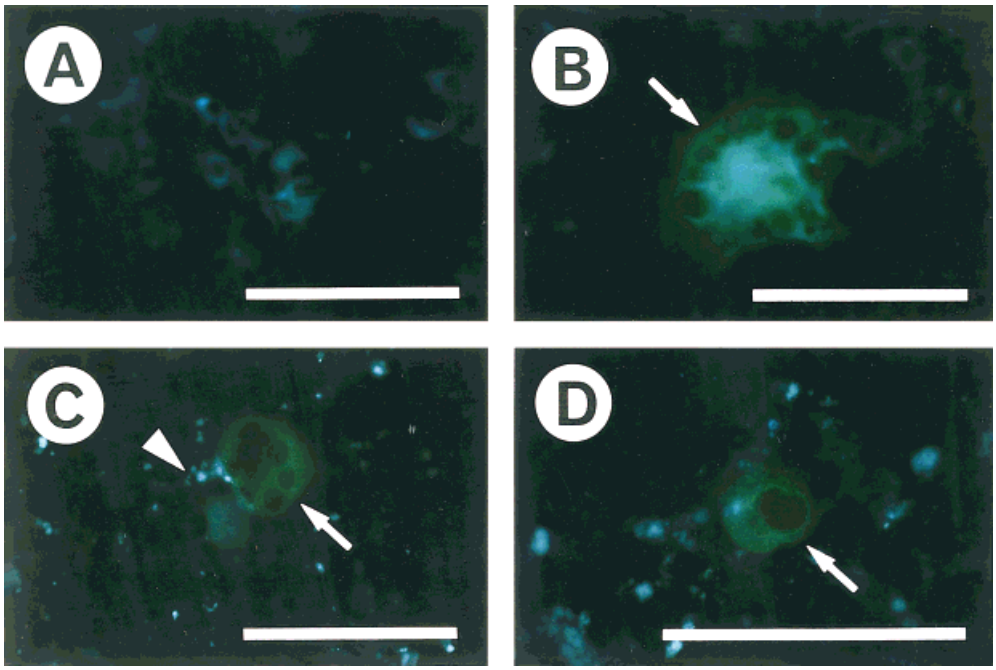

Fig. 4. Indirect immunofluorescence staining for HHV-6 gB of the VFVC-inoculated K-1034 cells 7 days after inoculation. The mock-infected **(A)** and VFVC-infected **(B, C, D)** K-1034 cells were examined by IFA as described in the text. Arrows indicate specific fluorescence in the cytoplasm of multinucleated giant cell (B, C) and single cell (D). Positive signals were not strong and there were giant cells with very weak fluorescence (a triangle). No fluorescence was seen in the mock-infected cells (A). Bars indicate 100 μm.

Thus, some cellular factors necessary for HHV-6A lytic infection may be significantly lower or some cellular factors inhibiting the virus replication may be more abundant in epithelial cells than in fully permissive cells such as HSB-2. This experimental infection system using the K-1034 cells and HHV-6A seems to be useful for determining the cellular factors critical for HHV-6 replication. A further study to identify the critical factors for HHV-6A replication is now in progress.

REFERENCES

Ablashi DV, Salahuddin SZ, Josephs SF, Imam F, Lusso P, Gallo RC, Hung C, Lemp J, Markham PD (1987): HBLV (or HHV-6) in human cell lines. Nature 329:207.

Ablashi DV, Josephs SF, Buchbinder A, Hellman K, Nakamura S, Llana T, Lusso P, Kaplan M, Dahlberg J, Memon S (1988a): Human B-lymphotropic virus (human herpesvirus-6). Journal of Virological Methods 21:29–48.

Ablashi DV, Lusso P, Hung CL, Salahuddin SZ, Josephs SF, Llana T, Kramarsky B, Biberfeld P, Markham PD, Gallo RC (1988b): Utilization of human hematopoietic cell lines for the propagation and characterization of HBLV (human herpesvirus-6). International Journal of Cancer 42:787–791.

Ablashi DV, Agut H, Berneman Z, Campadell-Fiume G, Carrigan D, Ceccerini-Nelli L, Chandran B, Chou S, Collandre H, Cone R, Dambaugh T, Dewhurst S, DiLuca D, Foa-Tomasi L, Fleckenstein B, Frenkel N, Gallo R, Gompels U, Hall C, Jones M, Lawrence G, Martin M, Montagnier L, Neipel F, Nicholas J, Pellett P, Razzaque A, Torrelli G, Thomson B, Salahuddin SZ, Wyatt L, Yamanishi K (1993): Human herpesvirus-6 strain groups: A nomenclature. Archives of Virology 129:363–366.

Agut H, Collandre H, Aubin JT, Guetard D, Favier V, Ingrand D, Montagnier L, Huraux JM (1989): In vitro sensitivity of human herpesvirus-6 to antiviral drugs. Research in Virology 140:219–228.

Aoyama T, Kurata T (1982): Viruses. In Kawamura A, Aoyama Y (eds.): "Immunofluorescence in medical sciences." Tokyo: University Tokyo Press, pp 101–157.

Burns WH, Sandford GR (1990): Susceptibility of human herpesvirus 6 to antivirals in vitro. Journal of Infectious Diseases 162:634–637.

Chen M, Popescu N, Woodworth C, Berneman Z, Corbellino M, Lusso P, Ablashi DV, DiPaolo JA (1994): Human herpesvirus 6 infects cervical epithelial cells and transactivates human papillomavirus gene expression. Journal of Virology 68:1173–1178.

DiLuca D, Katsafanas G, Schirmer EC, Balachandran N, Frenkel N (1990): The replication of viral and cellular DNA in human herpesvirus 6-infected cells. Virology 175:199–210.

Downing RG, Sewankambo N, Serwadda D, Honess R, Crawford D, Jarrett R, Griffin BE (1987): Isolation of human lymphotropic herpesviruses from Uganda. Lancet 2:390.

Fillet A-M, Reux I, Joberty C, Fournier J-G, Hauw J-J, Hoang PL, Bricaire F, Huraux J-M, Agut H (1996): Detection of human herpesvirus 6 in AIDS-associated retinitis by means of in situ hybridization, polymerase chain reaction and immunohistochemistry. Journal of Medical Virology 49:289–295.

Harnett G, Farr T, Pietroboni G, Bucens M (1990): Frequent shedding of human herpesvirus 6 in saliva. Journal of Medical Virology 30:128–130.

Hoshikawa N, Kojima A, Yasuda A, Takayashiki E, Masuko S, Chiba J, Sata T, Kurata T (1991): Role of the gag and pol genes of human immunodeficiency virus in the morphogenesis and maturation of retrovirus-like particles expressed by recombinant vaccinia virus: an ultrastructural study. Journal of General Virology 72:2509–2517.

Josephs SF, Salahuddin SZ, Ablashi DV, Schachter F, Wong-Staal F, Gallo RC (1986): Genomic analysis of human B-lymphotropic virus (HBLV). Science 234:601–603.

Kigasawa K, Soushi S, Tanaka Y, Obazawa H (1994): Morphological and chromosomal study of a human retinal pigment epithelial cell line. Japanese Journal of Ophthalmology 38:10–15.

Kigasawa K, Obazawa H, Soushi S, Tanaka Y (1995): Histochemical demonstration of acid phosphatase in a human retinal pigment epithelial cell line. Tokai Journal of Experimental and Clinical Medicine 20:31–35.

Lusso P, Salahuddin SZ, Ablashi DV, Gallo RC, Di Marzo Veronese F, Markham PD (1987): Diverse tropism of human B-lymphotropic virus (human herpesvirus 6). Lancet 2:743–744.

Lusso P, Markham PD, Tschachler E, Veronese FM, Salahuddin SZ, Ablashi DV, Pahwa S, Krohn K, Gallo RC (1988): In vivo cellular tropism of human B-lymphotropic virus (human herpesvirus-6). Journal of Experimental Medicine 167:1659–1670.

Lusso P, Malnati MS, Garzino-Demo A, Crowley RW, Long EO, Gallo RC (1993): Infection of natural killer cells by human herpesvirus 6. Nature 362:458–462.

Okuno T, Shao H, Asada H, Shiraki K, Takahashi M, Yamanishi K (1992): Analysis of human herpesvirus 6 glycoproteins recognized by monoclonal antibody OHV1. Journal of General Virology 73: 443–447.

Qavi HB, Green MT, SeGall GK (1989): Demonstration of HIV-1 and HHV-6 in AIDS-associated retinitis. Current Eye Research 8:379–387.

Qavi HB, Green MT, SeGall GK, Lewis DE, Hollinger FB (1992): Transcriptional activity of HIV-1 and HHV-6 in retinal lesions from AIDS patients. Investigative Ophthalmology & Visual Science 33:2759–2767.

Qavi HB, Green MT, Pearson G, Ablashi DV (1994): Possible role of HHV-6 in the development of AIDS retinitis. In vivo 8:527–32.

Qavi HB, Green MT, Lewis DE, Hollinger FB, Pearson G, Ablashi DV (1995): HIV-1 and HHV-6 antigens and transcripts in retinas of patients with AIDS in the absence of human cytomegalovirus. Investigative Ophthalmology & Visual Science 36:2040–2047.

Reux I, Fillet AM, Agut H, Katlama C, Hauw JJ, Le-Hoang P (1992): In situ detection of human herpesvirus 6 in retinitis associated with acquired immunodeficiency syndrome. American Journal of Ophthalmology 114:375–377.

Salahuddin SZ, Ablashi DV, Markham PD, Josephs SF, Sturzenegger S, Kaplan M, Halligan G, Biberfeld P, Staal FW, Kramarsky B, Gallo RC (1986): Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science 234:596–601.

Simmons A, Demmrich Y, Vista AL, Smith K (1992): Replication of human herpesvirus 6 in epithelial cells in vitro. Journal of Infectious Diseases 166:202–205.

Shiraki K, Okuno T, Yamanishi K, Takahashi M (1989): Phosphonoacetic acid inhibits replication of human herpesvirus-6. Antiviral Research 12:311–318.

Yamanishi K, Okuno T, Shiraki K, Takahashi M, Asano Y, Kurata T (1988): Identification of human herpesvirus-6 as a causative agent for exanthem subitum. Lancet i:1065–1067.

Yoshida M, Uno F, Bei Z-L, Yamada M, Nii S, Sata T, Kurata T, Yamanishi K, Takahashi M (1989): Electron microscopic study of a herpes-type virus isolated from an infant with exanthem subitum. Microbiology and Immunology 33:147–154.